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14. ABSTRACT The present study was initiated to provide US Navy with scientific data to help understand the environmental fate of RDX and HMX in marine environments. Two types of sediments were selected, one from a cold Atlantic site near Halifax, and another from a tropical Pacific site near Hawaii. Extensive analysis of sediments and water from both sites only showed the presence of DNT and TNT in two UXO sites from Hawaii. Indigenous microorganisms in both sites were found capable of degrading both RDX and HMX. Subsequent microbial characterization led to the isolation of several bacterial and fungal species capable of biodegrading the two nitramines. Kinetics determination and product analysis identified two major degradation routes. One route involved initial denitration followed by ring cleavage and a second route involved reduction of the N-NO <sub>2</sub> to the corresponding N-NO bonds prior to ring cleavage and decomposition. Some of the bacteria discovered were identified as novel species of Shewanella and are being sequenced for their genomes by DOE, US, making them the first explosives degraders to be sequenced for their genomes.						
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**(BIO)DEGRADATION OF RDX AND HMX IN  
MARINE/ESTUARINE WATER AND SEDIMENTS  
(GRANT N00014-03-1-0269)**

**Performing Organization**

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**Final Technical Report, Sept 2006**



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**GRANT #:** N000140310269

**PRINCIPAL INVESTIGATOR:** Dr. Jalal Hawari

**INSTITUTION:** Biotechnology Research Institute, NRCC

**GRANT TITLE:** (Bio)degradation of RDX and HMX in Marine/Estuarine Water and Sediments

**AWARD PERIOD:** 01 Jan 2003 – 30 June 2006

## **OBJECTIVE:**

To provide understanding on the microbial and enzymatic degradation of the two cyclic nitramine explosives, 1,3,5-hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), in marine and estuarine sediment by (i) studying the biodegradability of RDX and HMX in artificially contaminated field sediments; and (ii) identifying (bio)degradation products, kinetics and stoichiometries of degradation pathways using consortia and specific isolates from sediments. The data gathered will be used to provide insight into in-situ degradation in sediment.

## **APPROACH:**

- Sediments and water samples were collected at two marine locations: in Halifax (Canada) by Canadian Navy (FY02) and in Hawaii by the US Navy (FY03).
- Sediments were characterized for their physicochemical properties and for the presence of explosives and other co-contaminants.
- Microcosm experiments were conducted to evaluate the potential of indigenous bacteria to degrade RDX and HMX under various environmental and physiological conditions.
- RDX and HMX degraders, both fungi and bacteria, were isolated from Halifax and Hawaii sediments and identified using 18S/16S rRNA gene and biochemical properties.
- Microcosm experiments were conducted to evaluate the potential of isolated strains from both Hawaii and Halifax sediments to degrade RDX and HMX under anaerobic or aerobic conditions. Mineralization (liberated  $^{14}\text{CO}_2$ ) was monitored using  $^{14}\text{C}$ -RDX and  $^{14}\text{C}$ -HMX. Data from both types of sediments were compared.
- Enzymes were isolated from RDX and HMX degrading bacteria isolated from Halifax sediment.
- Key metabolites and degradation pathways of RDX and HMX were determined and data were used to provide insight into in-situ degradation in sediment.

## **ACCOMPLISHMENTS:**

### **1. Sampling of sediments**

Marine sediments were collected from two areas presenting a high risk of explosives contamination, one located in a cold Atlantic site and another located in a tropical Pacific site. In December 2002, sediment and water samples were collected by Canadian Navy in a deep sea-dump area located at depth of 215 m in Emerald Basin, around 50 nautical miles from Halifax harbor (Nova Scotia, Canada). In July 2003, four sediment samples were collected by US Navy

in two shallow unexploded ordnance (UXO) fields (UXO-1 and UXO-3), one shallow detonation field (UXO-5), and a shallow reference site (MIDREF-7) located offshore in Hawaii region (Hawaii, US). The sealed sediment samples were kept in a cold room at 4°C until use.

## 2. Characterization of sediments

Physicochemical properties that are relevant for the study of the microbial degradation of energetic chemicals are summarized in Table 1.

**Table 1. Physicochemical properties of the 5 sediment samples used in the present study**

	Halifax	Hawaii			
		UXO-1	UXO-3	UXO-5	MIDREF-7
pH	7.75	8.57	8.79	8.24	8.25
TOC (% dry wt.)	1.9	0.22	0.17	0.15	0.16
CEC (meq/100 g)	14.5	1.6	1.2	0.4	1.6
NH <sub>3</sub> (mg/kg dry)	7.7	60	21	9.5	61
Total S % (dry wt.)	0.36	0.20	0.17	0.20	0.20
Granulometry	Silt, Clay	Sand	Sand	Sand	Sand
Major metals (mg/kg dry)	Fe (28 000)	Ca (310 000)	Ca (350 000)	Ca (340 000)	Ca (270 000)
	Ca (24 000)	Mg (28 000)	Mg (21 000)	Mg (18 000)	Mg (19 000)
	Al (15 000)	Na (5 100)	Na (5 000)	Na (7 600)	Na (5 500)
	Na (10 000)	Sr (2 200)	Sr (2 400)	Sr (3 900)	Sr (2 500)
	Mg (8 800)	Fe (1 400)	Fe (1 900)	Fe (860)	Fe (440)
	K (5 300)				
Org. contaminants	Not detected	Not detected	Not detected	Not detected	Not detected
K <sub>d</sub> (RDX) (L kg <sup>-1</sup> )	1.3	Not measured	Not measured	Not measured	Not measured
K <sub>d</sub> (HMX)	5.2	Not measured	Not measured	Not measured	Not measured

Two analytical methods were developed for the analysis of energetic chemicals at trace levels in marine samples. We first developed a solid phase microextraction (SPME) technique for the recovery of explosives from aqueous samples using HPLC-UV detection. The SPME/HPLC method was then successfully used to analyze nine explosives (4-ADNT, 2,4-DNT, 3,4-DNT, TNT, 1,3-DNB, 1,3,5-TNB, RDX, HMX, tetryl) with method detection limits (MDL) ranging between 1 and 10 µg L<sup>-1</sup>, depending on the analyte. This SPME based method was shorter than the conventionally used solid phase extraction (SPE/HPLC-UV).

The second method coupling SPME with gas chromatography connected to an electron capture detector (SPME/GC-ECD) was applied to both seawater and marine sediment samples. Nine explosives (2-ADNT, 4-ADNT, 2,4-DNT, 2,6-DNT, TNT, 1,3-DNB, 1,3,5-TNB, RDX, tetryl) were analyzed with this method and gave MDLs ranging from 0.05 to 0.81 µg L<sup>-1</sup> in water and from 1 to 9 µg kg<sup>-1</sup> in dry sediment. With a smaller volume of aqueous sample required compared to SPE, SPME appeared attractive to analyze limited volumes of sediment pore-water. Moreover, the use of SPME eliminated interferences present in sediment extracts thus allowing the detection of the target analytes that were otherwise difficult to detect by direct injection into the GC.

These SPME based methods together with USEPA 8330 and USEPA 3535 methods were applied for the analysis of sediments from Halifax and Hawaii. No explosives or their degradation products (nitroso derivatives such as hexahydro-1,3-dinitro-5-nitroso-1,3,5-triazine (MNX), hexahydro-1-nitro-3,5-dinitroso-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)) were found in Halifax sample. In contrast, sediments collected in Hawaii showed the presence of 2,4-DNT (from 0 to 2.5 mg kg<sup>-1</sup>) and traces of TNT (< 10 µg kg<sup>-1</sup>) in samples UXO-1 and UXO-3 (Published in Monteil-Rivera et al. 2004 and 2005a).

### 3. Microbial degradation of RDX and HMX by indigenous mixed microbial population in Halifax and Hawaii marine sediments

*Biodegradation in Halifax sediment at 10°C.* Anaerobic indigenous microbial community in this sediment degraded RDX and HMX at 10°C with optimal mineralization achieved in the presence of glucose. Using [UL-<sup>14</sup>C]-RDX (66 µM) (in the presence of 4 µM non-labeled HMX) we obtained a <sup>14</sup>C mass balance distributed as follows: <sup>14</sup>CO<sub>2</sub>, 58 %; water soluble <sup>14</sup>C products, 27 %; acetonitrile extractable <sup>14</sup>C products, 1.9 %; and <sup>14</sup>C products covalently bound to the sediments and biomass, 8.9 %. In the case of [UL-<sup>14</sup>C]-HMX (4 µM) (in the presence of 66 µM non-labeled RDX), mineralization reached only 13-27 % after 115 days of incubation in the presence or absence of carbon sources. The disappearance of RDX was accompanied by the formation of HCHO and the mononitroso derivative, MNX, which only appeared transiently before evolving to HCHO and CO<sub>2</sub>. In the case of HMX the disappearance of the chemical was also accompanied by the formation of its mononitroso derivative. Based on products distribution, we suggested that RDX and HMX degraded *via* an initial reduction to the corresponding mononitroso derivatives followed by ring cleavage (Published in Zhao et al. 2004a).

*Biodegradation in Hawaii marine sediment at 21°C.* Anaerobic incubation of either RDX or HMX with sediment UXO-1 in marine medium (marine broth 2216, pH 6.5) at 21°C also led to their removal (101 µM of initial 103 µM RDX; 2.3 µM of initial 11.4 µM HMX) in 18 days. Degradation of RDX occurred through the transient formation of MNX, the ring cleavage product methylenedinitramine (MEDINA), and traces of DNX and TNX. Mineralization experiments were conducted using a nitramine-adapted mixed culture obtained by three consecutive sub culturing of the original sediment microbial population in RDX-containing or HMX-containing marine broth. The RDX-enrichment culture mineralized 69 % of [UL-<sup>14</sup>C] RDX in 25 days. Likewise, HMX-adapted mixed culture mineralized 57 % of [UL-<sup>14</sup>C]-HMX after 25 days (Published in Bhatt et al. 2005).

*Effect of oxygen and electron donors on RDX and HMX removal in three Hawaii sediments.* Both RDX and HMX were removed in UXO-3 and UXO-5 sediments when incubated under argon in the absence of any amendment. The removal of the two nitramines was lower when incubation was conducted with air in the headspace. Amendment of the two sediments with either glucose or yeast extract as carbon source and electron donor, increased their removal rates significantly. Abiotic controls showed some removal of RDX and HMX but much less than those observed in live sediment. HMX removal rates were found to be 6-20 times lower than those obtained for RDX under the same conditions, which is in line with the previously reported lower reactivity of the tetramer HMX (CH<sub>2</sub>NNO<sub>2</sub>)<sub>4</sub> as compared to the trimer RDX (CH<sub>2</sub>NNO<sub>2</sub>)<sub>3</sub>. RDX and HMX removal in the sediments was also accompanied by the formation of methylenedinitramine (MEDINA) confirming that the nitramines were metabolized by sediment indigenous microorganisms. Both nitramines were also removed in microcosms prepared with the MIDREF sediment sampled between UXO-3 and UXO-5 sites, with rates comparable to

those observed in UXO-3 and UXO-5. The above experiments clearly demonstrated that RDX and HMX would be mineralized under oxygen-limiting or anaerobic conditions by indigenous microorganisms present in subsurface environments including marine sediments (Zhao et al. 2006 submitted).

#### 4. Characterization of microbes involved in degradation of RDX and HMX in marine sediments

In both Hawaii and Halifax sediments, members of  $\gamma$ -proteobacteria were found as degraders of RDX. The isolated  $\gamma$ -proteobacteria from Halifax were psychrophilic *Shewanella* (HAW-EB1, HAW-EB2, HAW-EB3, HAW-EB4, and HAW-EB5), whereas those isolated from Hawaii were the mesophilic *Halomonas* (HAW-OC4), *Marinobacter* (HAW-OC1), and *Pseudoalteromonas* (HAW-OC2 and HAW-OC5) strains. None of the  $\gamma$ -proteobacteria was able to degrade HMX (Published in Zhao et al. 2004b; Bhatt et al. 2005).

Also in both Hawaii and Halifax sediments, Firmicutes (including Clostridiales), Fusobacteria, and  $\delta$ -proteobacteria were isolated as RDX and HMX degraders. In Hawaii sediments, four groups of anaerobic bacteria capable of removing both RDX and HMX were isolated and identified. Group I contained aerotolerant anaerobes and microaerophiles, group II contained facultative anaerobes while groups III and IV contained strictly anaerobic degraders. Using 16S rRNA genes, group I was assigned to a novel phylogenetic cluster of Clostridiales whereas groups II and III were most closely related to *Paenibacillus* and *Tepidibacter* of Firmicutes, respectively. Group IV bacteria were identified as *Desulfovibrio* of  $\delta$ -proteobacteria. In Halifax sediment, three obligately anaerobic RDX and HMX-degrading strains HAW-EB18, HAW-EB19 and HAW-EB21 were isolated and found to be affiliated to  $\delta$ -proteobacteria, Clostridiales and Fusobacteria, respectively (Zhao et al. submitted).

Furthermore, in UXO-1 Hawaii sediment, four fungal strains *Rhodotorula* sp. HAW-OCF1, *Bullera* sp. HAW-OCF2, *Acremonium* sp. HAW-OCF3 and *Penicillium* sp. HAW-OCF5, were isolated as RDX degraders under aerobic conditions. None degraded HMX (Published in Bhatt et al. 2006).

**Discovery of four new bacterial species.** We further characterized the phenotypic, genotypic and chemotaxonomic properties of psychrophilic RDX-degrading strains of *Shewanella* isolated from Halifax and found that they represent four novel species of *Shewanella* described as follows:

*Shewanella sediminis* sp. nov.: Strain HAW-EB3<sup>T</sup> contains lysine decarboxylase that is absent in other known *Shewanella* species, and distinguishes itself by the presence of arginine dehydrolase, ornithine decarboxylase and chitinase, and by its ability to oxidize and ferment *N*-acetyl-D-glucosamine. Strain HAW-EB3<sup>T</sup> grows on several carbon sources and shows distinctive fatty acid and quinone compositions. Both phenotypic and 16S rRNA gene phylogenetic cluster analyses demonstrated that HAW-EB3<sup>T</sup> belongs to the Na<sup>+</sup>-requiring group of *Shewanella* species. 16S rRNA gene sequence displayed  $\leq 97\%$  similarity to all known *Shewanella* species and was most similar to those of *Shewanella hanedai* and *Shewanella woodyi*. However, *gyrB* of strain HAW-EB3<sup>T</sup> was significantly different from those of other *Shewanella* species with similarities  $< 85\%$ . We proposed *Shewanella sediminis* sp. nov. with HAW-EB3<sup>T</sup> (= NCIMB 14036<sup>T</sup> = DSM 17055<sup>T</sup>) as the type strain (Zhao et al. 2005).

*Shewanella halifaxensis* sp. nov.: Aerobically-grown HAW-EB4<sup>T</sup> cells (marine broth, 10°C) contained C<sub>14:0</sub> (6%), iso-C<sub>15:0</sub> (12%), C<sub>16:0</sub> (20%), C<sub>16:1</sub> $\omega$ 7 (37%), C<sub>18:1</sub> $\omega$ 7 (7%), C<sub>20:5</sub> $\omega$ 3 (7%) as major membrane fatty acids, and Q7 (28.1%) and MK-7 (60.9%) as dominant respiratory

quinones, consistent with deep-sea species of *Shewanella*. This bacterium had a molar G + C content of 45% and was similar to *Shewanella* spp. in terms of 16S rRNA (93-99% similar) and *gyrB* (67.3-88.4% similar) gene sequences, with *Shewanella pealeana* being the most closely related species. DNA-DNA hybridization between strain HAW-EB4<sup>T</sup> and *S. pealeana*, showed a DNA homology of 17.9%, lower than the 70% species cut-off value, indicating that strain HAW-EB4<sup>T</sup> (= NCIMB 14093<sup>T</sup> = DSM 17350<sup>T</sup>) represents a novel species of *Shewanella*, proposed as *Shewanella halifaxensis* sp. nov. (Zhao et al. 2006a and b).

*Shewanella canadensis* sp. nov. and *Shewanella atlantica* sp. nov.: The two strains HAW-EB2<sup>T</sup> and HAW-EB5<sup>T</sup> were different from each other in their 16S rRNA (98.8% similar), *gyrB* (88.2% similar), and genomic DNA (22% similar). The 16S rRNA gene of strain HAW-EB2<sup>T</sup> or HAW-EB5<sup>T</sup> displayed high similarity (90-99.5%) to recognized species of *Shewanella*, but their *gyrB* genes showed a significant difference (79-86.8%) from known species of *Shewanella*. DNA-DNA hybridization showed that the genomic DNA of each of the two strains has a low relatedness to known species of *Shewanella* with a value of 41%, lower than the species cut-off value for bacteria. In comparison to species of *Shewanella*, each of the two strains was also unique in some phenotypic properties such as activity of gelatinase and lipase, and capability to metabolize organic acids and sugar. Both strains HAW-EB2<sup>T</sup> and HAW-EB5<sup>T</sup> utilize malate, valerate, peptone and yeast extract as sole carbon and energy sources. The major membrane fatty acids of the two strains were C<sub>14:0</sub>, iso-C<sub>15:0</sub>, C<sub>16:0</sub>, C<sub>16:1</sub>ω7, C<sub>18:1</sub>ω7 and C<sub>20:5</sub>ω3 and their major quinones were Q7, Q8 and MK-7. Strain HAW-EB2<sup>T</sup> (NCIMB14238) was proposed as the type strain of *Shewanella canadensis* sp. nov. Strain HAW-EB5<sup>T</sup> (NCIMB14239) was proposed as the type strain of *Shewanella atlantica* sp. nov. (Zhao et al. submitted).

## 5. Chemotaxis in RDX and HMX degradation in marine environment

When present in sediments, RDX and HMX, which are weakly soluble in water, may be attached to solid surfaces and/or trapped in pores. For efficient bioremediation of these explosives, the microorganism(s) must access them. Bacterial chemotaxis is one such process that brings microbes closer to the contaminated sites and thus enhances the rate of biodegradation. Using the technique we isolated an obligate anaerobic bacterium *Clostridium* sp. strain EDB2 from Halifax sediment. The strain was motile with numerous peritrichous flagella and demonstrated chemotactic response towards RDX, HMX and NO<sub>2</sub><sup>-</sup>. This finding is important because it provides evidence for the presence of chemotactic bacteria in deep sediment environments, which allows access of these microbes to explosives even if immobilized in sediments (Published in Bhushan et al. 2004).

## 6. Effect of iron (III), humic acid, and anthraquinone-2,6-disulphonate on biodegradation of cyclic nitramines by the chemotactic bacterium *Clostridium* sp. EDB2.

Bacterial growth and biodegradation of RDX and HMX were stimulated in the presence of Fe(III), humic acid (HA), and anthraquinone-2,6-disulphonate (AQDS), suggesting their utilization as redox-mediators to cyclic nitramines by strain EDB2. We suggested that strain EDB2 degraded RDX and HMX by 1) direct degradation of the chemicals; 2) indirect degradation by reducing Fe(III) to produce reactive-Fe(II); and 3) indirect degradation by reducing HA and AQDS which act as electron-shuttles to transfer electrons to the cyclic-nitramines. Since microbial reduction of iron and humic substances occurs on a large scale in subsurface environments such as sediments, estuaries and groundwater, such environments

would be ideal for accelerating the cleanup of cyclic nitramine energetic chemicals (Published in Bhushan et al. 2006).

### 7. Enzymes involved in microbial degradation of RDX and HMX

From *Clostridium* sp. strain EDB2, an obligately anaerobic bacterium chemotactic to RDX and HMX from Halifax sediment, an NADH-dependent RDX and HMX-degrading enzyme (56 kDa) was extracted. Sequence comparisons revealed that N-terminal amino-acid of the purified enzyme was homologous to a variety of dehydrogenases from several *Clostridium* spp. The purified putative hydrogenase degraded RDX and HMX at rates of 13 and 5.5 nmol h<sup>-1</sup> mg<sup>-1</sup> protein, respectively, under anaerobic conditions. Both RDX and HMX produced nitrous oxide and formaldehyde as common degradation products.

We also investigated HMX (RDX) degradation by a commercial enzyme Xanthine oxidase (XO). Xanthine oxidase (XO) catalyzed biotransformation of HMX at rates of 1.6 ± 0.2 and 10.5 ± 0.9 nmol h<sup>-1</sup> mg protein<sup>-1</sup>, under aerobic and anaerobic conditions, respectively. Biotransformation of HMX was accompanied with the formation of NO<sub>2</sub><sup>-</sup>, MEDINA, 4-NDAB (4-nitro-2,4-diazabutanal), HCHO, N<sub>2</sub>O, HCOO<sup>-</sup>, and NH<sub>4</sub><sup>+</sup>. The product distribution gave carbon and nitrogen mass-balances of 91 and 88 %, respectively. A comparative study with native-, deflavo- and desulfo-XO and site-specific inhibition studies showed that HMX biotransformation occurred at the FAD-site of XO. Nitrite stoichiometry revealed that an initial single *N*-denitration step was sufficient for the spontaneous decomposition of HMX (published in Bhushan et al. 2003). In another study we found that crude extract of *Clostridium bifermentans* strain HAW, isolated previously from anaerobic sludge, degraded RDX and HMX in the presence of several e-donors such as H<sub>2</sub>, NADH or NADPH, suggesting the involvement of multiple enzymes including hydrogenase or NAD(P)H-dependent reductase. Similarly, RDX and HMX degradation were accompanied by concurrent release of nitrite without the formation of the nitroso derivative(s) (Published in Zhao et al. 2004c).

RDX degradation enzymatic activity was also found in crude cellular extract prepared from the above mentioned *Shewanella halifaxensis* HAW-EB4, an obligately respiratory and psychrophilic species found in Halifax sediment. RDX-degradation activity has been found to be favorably induced under TMAO-reducing condition. Preliminary enzyme assays showed the RDX degradation activity is NADH-dependent. Thus far all enzymes or crude enzyme extract examined were found to metabolize RDX or HMX via a 1e<sup>-</sup> transfer process leading to denitration although 2e<sup>-</sup> reduction of N-NO<sub>2</sub> to the corresponding N-NO was also observed in whole cells of studied microorganisms. To address this issue, we are currently characterizing both the 2e<sup>-</sup> and 1e<sup>-</sup> transfer processes of nitro reductase using *S. halifaxensis*. This task will be studied in more details in the new ONR project N000140610251.

### 8. Product distributions, stoichiometry and degradation pathways of RDX and HMX by representative marine isolates

*Anaerobic biodegradation of RDX.* Psychrophilic  $\gamma$ -proteobacteria *Shewanella* sp. HAW-EB1 - EB5 mineralized 26-45.2 % of RDX (92  $\mu$ M) in 82 days of incubation at 10°C under oxygen-limited conditions. Removal of RDX was accompanied by the formation of all three nitroso derivatives, with MNX being the major one. Using resting cells, the two ring cleavages products previously identified in our group, methylenedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (4-NDAB), which eventually disappeared to produce N<sub>2</sub>O and HCHO (mineralized as determined by liberated <sup>14</sup>CO<sub>2</sub>), were detected (Published in Zhao et al. 2004a).

Likewise the mesophilic  $\gamma$ -proteobacteria marine bacteria isolated from Hawaii sediment UXO-1 including *Halomonas* (HAW-OC4), *Marinobacter* (HAW-OC1), *Pseudoalteromonas* (HAW-OC2 and HAW-OC5) degraded RDX similarly to  $N_2O$  and HCHO via the intermediate formation of MNX and MEDINA (Published in Bhatt et al. 2005).

*Anaerobic biodegradation of HMX.* HMX degradation ability was found in obligately anaerobic Clostridiales and Fusobacteria and sulfate reducing  $\delta$ -proteobacteria. Clostridiales and  $\delta$ -proteobacteria isolated from Hawaii sediments metabolized HMX to  $N_2O$  and HCHO via the intermediary formation of methylenedinitramine (MEDINA). In addition, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine together with trace amounts of the di-, and tri-nitroso derivatives of HMX were detected with all isolates. The respiratory  $\delta$ -proteobacteria strain MIDREF-29 isolated from Hawaii sediment (MIDREF site) is the first anaerobic bacteria that mineralized appreciable amounts of HMX. Using uniformly labeled  $^{14}C$ -[HMX], strain MIDREF-29 mineralized 26.8% of HMX in 308 d as determined by liberated  $^{14}CO_2$ . Negligible HMX mineralization was observed with the Clostridiales (Zhao et al. submitted).

*Aerobic biodegradation of RDX by marine fungus Acremonium sp..* Aerobic degradation was only found in marine fungi. When RDX (100  $\mu M$ ) was incubated with resting cells of *Acremonium* sp. under aerobic condition, we detected methylenedinitramine (MEDINA),  $N_2O$  and HCHO. Also we detected hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) together with trace amounts of hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Under the same conditions MNX produced  $N_2O$  and HCHO together with trace amounts of DNX and TNX, but we were unable to detect MEDINA. TNX did not degrade with *Acremonium* sp. (published in Bhatt et al. 2006).

In general, in all studied microorganisms we identified two major pathways for degradation of RDX and HMX, one involved 2e reduction of  $-NO_2$  to  $-NO$  to produce the corresponding nitroso derivatives and the second involved 1e reduction of nitro group leading to denitration followed by ring cleavage and decomposition to eventually produce (HCHO,  $CO_2$ ,  $N_2O$  etc.). These findings are consistent with our previous observation using anaerobic sludge isolates such as *Clostridium bifermentans* HAW-1.

## CONCLUSIONS:

Marine sediment and water samples were collected from two sites, one at Emerald Basin in Halifax (10°C and 215 m below surface water) and another at Oahu Island in Hawaii (21°C, approx 10-21 m below water surface). Extensive analysis of sediments from both sites did not show the presence of any explosives except two UXO sites in Hawaii which showed the presence of DNT and TNT. Indigenous microorganisms in both sites showed potential for the degradation of both RDX and HMX. Subsequent microbial characterization led to the isolation of several bacterial and fungal microorganisms capable of degrading the two cyclic nitramines. Extensive kinetics determination and product analysis identified two major pathways for the degradation of the two nitramines. One pathway involved initial denitration followed by ring cleavage and a second involved reduction of the  $N-NO_2$  to the corresponding  $N-NO$  prior to ring cleavage and decomposition. Some of the bacteria discovered were identified as novel species of *Shewanella* and are presently being sequenced for their genomes by DOE, US. The genome sequencing will be reported within the second project with ONR to help determine the genes and enzymes responsible for the degradation of various explosives.

## **SIGNIFICANCE:**

The discovery of microorganisms capable of biodegrading RDX and HMX in marine sediment and the identification of key metabolites improved significantly our understanding of the fate of explosives in marine and estuarine water sediments. The knowledge of metabolites will allow us to develop chemical sensors for the detection of the key marker products methylenedinitramine and 4-nitro-2,4-diazabutanal to monitor and assess natural attenuation. In fact 4-nitro-2,4-diazabutanal has already been detected by our laboratory in groundwater contaminated with RDX near an ammunition plant in Iowa, US. The second major significant contribution of the present work is the potential development of biomarkers to search and identify microorganisms (gene mining) capable of degrading explosives at other contaminated marine environments. Genomics offers powerful tools to study the gene and enzymes in biodegradation by pure culture or whole microbial community. Following the submission of a proposal prepared in collaboration with members of the *Shewanella* Federation, the Department of Energy (DOE) accepted to sequence the genome of four strains of *Shewanella* including the two Halifax RDX-degrading strains HAW-EB3<sup>T</sup> and HAW-EB4<sup>T</sup> (DOE website <http://jgi.doe.gov/sequencing/DOEmicrobes2006.html>). The discovered *Shewanella* apparently are the first microbes to be sequenced for their genomes as explosives degraders.

## **PATENT INFORMATION:**

## **AWARD INFORMATION:**

1. Biotechnology Research Institute, Director General Award for international visibility (2003).
2. TTCP-WTP4 France Beaupré Outstanding Service Award (Co-recipient), UK (2005).  
TTCP stands for The Technical Cooperation Program of International Defence Departments and Ministries of Canada, UK, Australia, USA, and N.Z.
3. Biotechnology Research Institute, Director General Outstanding achievement award for the discovery of the new species of *Shewanella* and the genome sequencing of the isolates (Dec 2005).

## **PUBLICATIONS and ABSTRACTS:**

### **Publications in peer reviewed journals:**

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18. Zhao J-S, Manno D, Thiboutot S, Ampleman G, Hawari J. *Shewanella canadensis* sp. nov., *Shewanella atlantica* sp. nov., two novel MnO<sub>2</sub> and hexahydro-1,3,5-trinitro-1,3,5-triazine-reducing psychrophilic species of marine bacteria. (Submitted)
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